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Biochemical Studies of the Tracheobronchial Epithelium

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Tracheobronchial epithelium has been a focus of intense investigation in the field of chemical carcinogenesis. We have reviewed some biochemical investigations that have evolved through linkage with carcinogenesis research. These areas of investigation have included kinetics of carcinogen metabolism, identification of carcinogen metabolites, levels of carcinogen binding to DNA, and analysis of carcinogen–DNA adducts. Such studies appear to have provided a reasonable explanation for the susceptibilities of the respiratory tracts of rats and hamsters to carcinogenesis by benzo(a)pyrene. Coinciding with the attempts to understand the initiation of carcinogenesis in the respiratory tract has also been a major thrust aimed at effecting its prevention both in humans and in animal models for human bronchogenic carcinoma. These studies have concerned the effects of derivatives of vitamin A (retinoids) and their influence on normal cell biology and biochemistry of this tissue. Recent investigations have included the effects of retinoid deficiency on the synthesis of RNA and the identification of RNA species associated with this biological state, and also have included the effects of retinoids on the synthesis of mucus-related glycoproteins. Tracheal organ cultures from retinoid-deficient hamsters have been used successfully to indicate the potency of synthetic retinoids by monitoring the reversal of squamous metaplasia. Techniques applied to this tissue have also served to elucidate features of the metabolism of retinoic acid using high pressure liquid chromatography. In brief, formidable strides have been made in biochemistry specific to this important target tissue, despite the inability to acquire tracheobronchial epithelium in large quantities.

Introduction

The tracheobronchial epithelium is a primary interface with the environment, its constituents and its contaminants. It is, then, not surprising that this tissue gives rise to more fatal malignancies than any other tissue in the human body. Cancers of the respiratory tract cause 14% of cancer deaths in women and 34% in men (*1*). Because the vast majority of lung cancers are, in reality, cancers of the cells lining the large airway passages (*2*), a concerted effort has been directed at achieving experimental models of human lung cancers. The intratracheal instillation technique of Saffiotti et al. (*3*), the heterotopic tracheal graft model of Nettesheim et al. (*4*) and the localized instillation technique of Schreiber et al. (*5*) all produce cancers with histologic similarities to human bronchogenic epidermoid carcinoma. This review will consider the development of experimental methods and the pursuit of two lines of investigation which have evolved from these experimental models of lung cancer. The first of these investigative issues concerns the activation of carcinogens in this target tissue, and the second relates to the action of a class of agents, retinoids, that may have promising cancer chemopreventive properties in this tissue.

We review here findings of some current investigations in which epithelial cells of the tracheobronchial airways have been used as a source for various biochemical analyses. These investigations have included carcinogen metabolism, binding and adduct analysis, glycoprotein biosynthesis, RNA synthesis, bioassay of retinoids and retinoic acid metabolism.

Biochemical studies in the tracheobronchial epithelium appear, upon first consideration, to be a rather limited proposition because of the small quantity of tissue that is available. However, the studies mentioned below attest to the fact that it is possible to perform some sophisticated analyses in this tissue.

Methods for Biochemical Study of Tracheobronchial Epithelium

The tracheobronchial epithelium is far more difficult to use for biochemical studies than solid organs. The cells comprising this tissue are those which line the major pulmonary airways as well as connective tissue, smooth muscle, and cartilage. Those biochemical characteristics specific to the epithelial cells can be appreciated if these cells can be separated from their supporting elements. Yet, such a procedure yields small

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quantities of cells, using many animals, a large expenditure of time, and considerable cost. Nonetheless, biochemical methods have been developed for studies with this tissue (Table 1).

Initially, studies of biochemical processes utilized the intact trachea in large laboratory animals. Wardell et al. (*6*) studied tracheal mucus production in dogs by collecting secretions from grafted tracheas. Excised segments of the trachea were implanted subcutaneously to form closed pouches, or sewn such that the lumen of the trachea communicated with the outside through a tracheocutaneous fistula. Secretions were collected from the heterotopically placed tracheas and analyzed. Recently, studies of glycoprotein biosynthesis by Clark and Marchok (*7*) utilized a modification of this graft technique but with rat tracheas. These investigators were able to collect milligrams of tracheal mucus for subsequent analyses.

Table 1. Examples of methods used for tracheobronchial biochemistry.

Procedure	Species	References
Isolation of epithelial cells		
Mechanical isolation	Hamster	(*8,13*)
Enzymatic dissociation	Hamster	(*11,29*)
	Rat, mouse, rabbit	(*32*)
Isolation of cell components		
Nuclei	Hamster	(*8*)
Microsomes	Hamster	(*13*)
Cell homogenates	Hamster	(*13,14,20*)
Macromolecule isolation		
DNA	Hamster	(*24*)
RNA	Hamster	(*73,88*)
Glycoproteins	Hamster	(*11,84,89,90*)
	Rat	(*7,10,76,77*)
	Dog	(*6*)
	Rabbit	(*32,91*)
Glycerolipids	Rat	(*92*)
Carcinogen metabolism		
Carcinogen–DNA binding		
BP	Hamster	(*13,14,24,28*)
BP	Rat	(*14,28*)
BP	Bovine	(*28,93*)
BP	Human	(*15,28,59*)
BP	Mouse	(*28*)
DMBA	Human	(*20*)
Nitrosamines	Human	(*54*)
TLC analysis		
BA, 7-MeBA, BP	Human, rat, hamster	(*36*)
BP	Human, rat, hamster	(*17–19*)
DMBA	Rat	(*44*)
HPLC analysis		
BP	Human	(*20,28*)
BP	Hamster	(*13,14,28*)
BP	Rat	(*14,17,28*)
BP	Mouse	(*28*)
Carcinogen–nucleoside adduct analysis		
BP	Bovine	(*93*)
BP	Human	(*59*)
BP	Hamster, rat, mouse	(*28*)
Aflatoxin B_1	Human	(*55*)
DNA repair		
Unscheduled synthesis	Rat	(*94*)
BP–DNA adduct removal	Hamster	(*33*)
Retinoic acid metabolism	Hamster	(*34,35,37,80*)

To study subcellular and macromolecular elements specifically originating from the epithelial cells, it was necessary to have a technique to isolate these cells from the rest of the trachea. Smith et al. (*8*) were able to recover a tracheal cell population by scraping the epithelium from the tracheal surface of Syrian hamsters. Nuclei isolated from the tracheal cells were morphologically intact and competent at the synthesis of RNA. Based on earlier observations that tracheal organ cultures could be maintained *in vitro* with reasonable morphologic preservation, Kaufman et al. (*9*) showed that excised hamster tracheas incorporated radioactively labeled macromolecular precursors from culture medium during relatively brief periods of incubation *in vitro*. Precursors of protein, RNA and DNA were incorporated into these macromolecules within tracheal epithelial cells and were analyzed in cells scraped from the tracheas after the incubation period. Autoradiographic studies performed simultaneously with biochemical studies showed that thymidine was largely incorporated into basal cells, uridine was most actively incorporated into the nuclei of basal cells and ciliated cells, whereas leucine was most actively incorporated into mucous cells. These initial studies also showed that macromolecules could be sufficiently labeled in this manner to permit more sophisticated biochemical studies.

Subsequently, Bonanni et al. (*10,11*) showed that when relatively stable macromolecular products such as glycopeptides were the object for study, such products could be isolated from tracheal epithelial cells that were enzymatically dissociated from the underlying supportive structures by incubation with hyaluronidase. Even smaller quantities of tissue than those used above apparently have been sufficient for studies of gel-electrophoretic analyses of LDH isozymes in the bronchi of rats exposed intratracheally to benzo(a)pyrene (BP). In these studies, bronchial mucosa was isolated from rats by smearing the epithelium onto a slide, freeze-drying the slide, and scraping off the epithelial cell residue (*12*).

Thorough studies of carcinogen metabolism in tracheobronchial epithelium also became feasible as the result of organ culture of this tissue. Prolonged and reproducible exposures of the epithelium directly to carcinogens were possible, and since no other parts of the respiratory tract were present, the specific features of metabolism in this tissue could be evaluated. Studies of carcinogen metabolism have progressed further through the development of methods for preparation of epithelial cell homogenates from this tissue and isolation of microsomes. Typical kinetic evaluations of BP monooxygenase have been feasible with microsomal

fractions from rat and hamster tracheas (*13,14*), and the activity of the monooxygenase was detectable in homogenates of cells scraped from human bronchus (*15,16*). Specific products of the biotransformation of radiolabeled carcinogens have been separated and identified by thin layer and high-pressure liquid chromatography (*13,14,17–23*). The binding of the carcinogen BP to DNA was quantitated in cells isolated from hamster tracheas by the scraping technique (*24*). DNA was purified from tracheal cells by phenol extraction, solvent extraction of unreacted BP, enzymatic removal of protein and RNA, followed by banding at equilibrium in centrifugally generated CsCl gradients. These techniques were applied to the isolation of DNA from human bronchial tissues (*15,20,25–27*). Though the quantities of DNA isolated are often on the order of micrograms, this has proven sufficient to allow BP–DNA adduct analyses in a variety of species including hamsters, rats, mice, bovines and humans (*28*).

With the advent of cell culture techniques for respiratory epithelial cells (*29–32*), defined populations of cells from this tissue have been evaluated for their metabolic characteristics with respect to BP (*18*). Recently, Eastman et al. (*33*) utilized hamster tracheal cells in monolayer culture to perform the first reported studies of repair of carcinogen–DNA adducts in respiratory epithelial cells.

Sporn and colleagues (*34–40*) have been studying the mechanism of action of retinoids in the respiratory epithelium. Organ cultures of the tracheobronchial epithelium from hamsters were found suitable as exquisitely sensitive bioindicators of the potency of retinoids. Studies on the metabolism of retinoic acid and its active intermediates (*34,35*) have proceeded somewhat like studies of carcinogen metabolism with metabolites of retinoic acid extracted from culture medium by organic solvents and analyzed by HPLC.

The preceding discussion of biochemical methods employed for studies of tracheobronchial epithelium indicates the progress that has been made in this area of biochemistry. The following two sections illlustrate more thoroughly two areas of research on the biochemistry of the tracheobronchial tract that have shown notable progress.

Carcinogen Metabolism in Tracheobronchial Epithelium

Biological Evidence of Metabolism of Polynuclear Hydrocarbons in Respiratory Epithelium of Rodents

The lung consists of at least 40 cell types (*41*). A proportionately large share of these cells provides structural support for lung architecture and lines the capillary network which interfaces the air spaces to the blood supply. It is the cells which line the trachea and bronchi which are at manifestly greater risk for the development of lung cancer, but the cells of the tracheobronchial epithelium comprise a minority of the total cell population within the lung. Studies of carcinogen activation in whole lung homogenates or subcellular fractions thereof, do not directly reveal the characteristics of polynuclear hydrocarbons (PNH) metabolism in the cells most vulnerable to carcinogens, the cells lining the tracheobronchial airways.

BP, a ubiquitous carcinogen and constituent of cigarette smoke, serves as a most well characterized marker for the presence of PNH in the environment. Until recently there has been little information acquired concerning BP metabolism in respiratory tract tissues where this agent may exert a carcinogenic effect *in vivo*.

Wattenberg and Leong (*42*) in 1962 attempted to identify and localize sites of PNH metabolism in frozen sections of rodent tissues using a histofluorescent assay for "pyridine nucleotide dependent polycyclic hydrocarbon metabolizing systems." They were unable to demonstrate that respiratory epithelium metabolized BP since these cells lacked fluorescent characteristics of BP metabolites, but metabolic capacity was observed in alveolar walls. However, studies of respiratory carcinogenesis by Saffiotti et al. (*3*) in which BP–ferric oxide was administered intratracheally to hamsters implied that the trachea and bronchi could metabolize or, at least, respond to this carcinogen by virtue of the development of tracheobronchial cancers following such carcinogen treatments. Theoretically, enzymatic activation could have occurred elsewhere in the respiratory tract and activated metabolites could have entered tracheal or bronchial cells by diffusion. Alternatively, pulmonary macrophages which scavenged the instilled carcinogen-laden particulate material could have released active metabolites (*43,44*). More direct evidence of the capacity for carcinogen metabolism in tracheal epithelium was provided by Dirksen and Crocker (*45*) and by Palekar et al. (*46*). These investigators observed that tracheal organ cultures of rat or hamster maintained in medium containing 7,12-dimethylbenz(a)anthracene (DMBA), benz(a)anthracene (BA), BP, and 3-methylcholanthrene (MCA) exhibited morphologic changes of hyperplasia, metaplasia and other general cytotoxic reactions. This observation narrowed the likely site of activation since other elements of the respiratory tract were absent. The alterations in this tissue, therefore, were most probably related to the conversion of the PNH to toxic metabolites directly in tracheal cells. These results have been reproduced and extended in more recent years by Lane and Miller (*47,48*), and by Mossman and Craighead (*49*) with BP and MCA, respectively.

More biologically integrated studies of carcinogen activation in respiratory epithelium began to explore animal models for human lung cancer. Biochemical and anatomic factors which contributed to susceptibility to carcinogen-induced neoplasia in animals were character-

ized and considered with respect to their relevence to humans. Studies in hamsters with the BP–ferric oxide model of Saffiotti et al. (*3*) demonstrated that the trachea was an ideal target tissue for biochemical investigations. Subsequent studies have used human respiratory tissues in explant culture.

Carcinogen Metabolism and DNA Binding in Rodent Respiratory Tissues

Kaufman et al. (*24*) gave the first biochemical evidence of the capacity of tracheal epithelium to metabolize BP. Excised hamster tracheas were incubated in tissue culture medium containing [^{3}H]BP, and epithelial cells were subsequently obtained by scraping the mucosal surface of tracheas and the DNA from these cells was isolated and purified in CsCl gradients. The DNA had radioactivity associated with it which resisted repeated ether extraction and was therefore attributed to covalently bound [^{3}H]BP. Prior treatment of hamsters with intratracheal BP–ferric oxide enhanced the binding of [^{3}H]BP to DNA in tracheal epithelial cells. This observation demonstrated substrate inducible metabolism, a feature of cytochrome P-450-mediated reactions. That this enzyme complex was involved in the carcinogen binding was confirmed by the observation that 7, 8-benzoflavone (7,8-BF) inhibited the binding of [^{3}H]BP to DNA in tracheas from BP–ferric oxide-pretreated animals but not in tracheas from control animals. This flavone inhibits the metabolism of PNH catalyzed by liver microsomes obtained from rats pretreated with an intraperitoneal injection of MCA. Autoradiographic analysis (*15,27*) demonstrated morphologically that [^{3}H]BP was bound to cytoplasmic and nuclear constituents primarily in the epithelial cells. The extent of binding of [^{3}H]BP in these autoradiographic studies was reduced by co-incubation with 7,8-BF; this finding was consistent with the biochemical studies.

Harris et al. (*20*) used a fluorometric assay (*50*) to measure aryl hydrocarbon hydroxylase (AHH) activity in homogenates of cells scraped from bronchial organ cultures. The radiometric assay of Hayakawa and Udenfriend (*51*) was used to measure metabolites released into the organ culture medium from explants of bronchial epithelium (*20,43*). The fluorometric assay sensitively measures phenolic products of BP metabolism, and the assay of Hayakawa and Udenfriend (*51*) measures the production of tritiated water resulting from oxygenated ^{3}H-hydrocarbon metabolites which have undergone NIH shift concomitantly with tritium loss upon formation. These assays offered a measure of the formation of BP phenols and quinones by tracheal tissue with the greatest sensitivity then available. These methods, however, did not permit the various products to be distinguished; some products, for example, dihydrodiols, retain tritium upon formation, and their presence was undetected.

Pal et al. (*22*) were the first to separate the products of metabolism of PNH to further characterize these metabolic processes in tracheobronchial epithelium. They showed that thin-layer chromatography (TLC) could be used to separate and identify phenol and dihydrodiol metabolites of BA, 7-MeBA, and BP produced by organ cultures of human bronchus and by rat and hamster trachea. Cohen et al. (*19*) used organ cultures of tracheas, bronchi and peripheral lung from rats, hamsters and humans as well as isolated perfused rat and hamster lung in studies of BP metabolism. BP metabolites, separated by TLC showed qualitative similarities in metabolite patterns between tissues and species. Qualitative differences were found between metabolism in hamster lung fragments and in tracheas and bronchi; this generally and perhaps fortuitously corresponded to the relative susceptibility of these tissues to tumorigenesis by BP in these tissues. Although a low level of BP metabolism was detected in lung tissue, this was of uncertain significance because early signs of necrosis (darkened nuclei) were seen in histologic sections of the lung fragments, and this would account for the low level of metabolism. In another report, Cohen et al. (*17*) identified an ethyl acetate-soluble conjugate between sulfate and monohydroxy BP that was a major metabolite in organ cultures of respiratory tissues. Despite the identification of products of metabolism, these observations offered only a limited view of carcinogen metabolism in respiratory tract tissues for two reasons. First, TLC methods offered qualitative identification of products but quantitative measurements were not readily made. Second, since the underlying microsomal metabolism had not been evaluated, the pattern of metabolites, particularly those conjugated forms released into the organ culture medium, could not be related to the primary products of BP metabolism by microsomes.

The assay for BP metabolism developed by DePierre et al. (*52*) quantitated all of the products formed with great improvement in sensitivity and precision. This offered the possibility for direct measurement of BP metabolism by microsomes from tracheobronchial epithelium. Microsomes were isolated by Mass and Kaufman (*13,14*) from epithelial cells scraped from rat or hamster tracheas; the yield of microsomal protein was about 5 to 10 μg per trachea. By using this more sensitive radiometric assay, metabolism of BP by microsomes from hamster tracheas was detectable; furthermore, Lineweaver-Burk plots were prepared and K_m and V_{max} values were estimated. Preincubation of tracheas with BP prior to isolation of microsomes doubled the V_{max} of the monooxygenase; nonetheless the activity of tracheal microsomes was 50-fold lower than the activity of microsomes from the livers of rats pretreated with IP injections of MCA. K_m values for microsomes from hamsters fell between 1 and 2 μM, suggesting that the monooxygenase has a high affinity for BP. Similar studies with microsomes from rat tracheas never detected metabolism, presumably because the activity of benzopyrene monooxygenase in rat

tracheal microsomes is below the limit of detection of this assay. The activity was detected in 10,000*g* supernatants of rat tracheal cell homogenates, but only if the trancheas were preincubated with BP prior to cellular isolation. The metabolic activity of supernatants from rat and hamster tracheal cell homogenates are compared in a Lineweaver-Burk plot. (Fig. 1) The BP-induced component of the mixed-function oxidase in rat tracheal cells was half that of hamsters; however, the K_m values, spanning the 1 to 2 μM range, were essentially equivalent. Titration of 7,8-BF effects on benzopyrene monoxygenase from 10,000*g* supernatants of hamster tracheas (Fig. 2) showed results like those previously seen for DNA binding (*24*). BP pretreatment increased the sensitivity of benzopyrene monooxygenase to 7,8-BF.

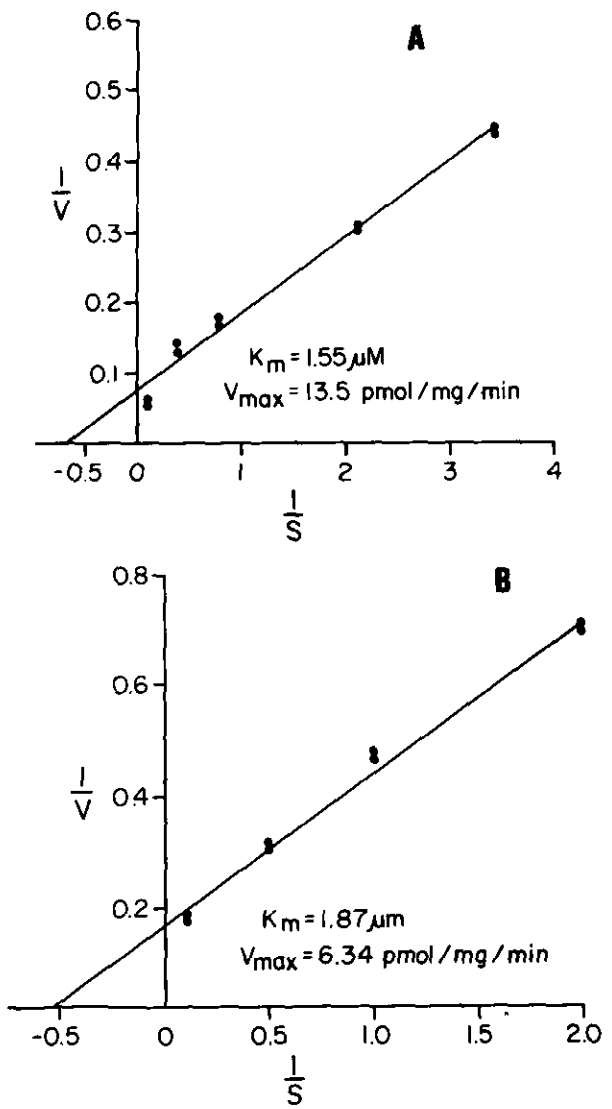

FIGURE 1. Lineweaver-Burk plot for benzopyrene monooxygenase activity in supernatants of tracheal epithelial cell homogenates centrifuged at 10,000*g*: (*A*) BP-pretreated hamster tracheas: (*B*) BP-pretreated rat tracheas. Ordinate expressed as min/pmole; abscissa, inverse substrate concentration expressed as 1/μM.

The adoption of HPLC analysis of metabolites produced by hamster trancheal microsomes showed that about 50% of the metabolites produced by hamster tracheal microsomes cochromatograph with quinone derivatives of BP (Fig. 3). BP-phenols (3-OH and 9-OH) comprised the second major class of metabolites and these were well separated from dihydrodiols (diols) of BP, the next most prevalent class of oxygenated products. Metabolites produced by microsomes isolated from BP-induced rat tracheal organ cultures had HPLC profiles that differed from those produced by hamster tracheal microsomes (Fig. 4). Rat tracheal microsomes produced almost exclusively 3-OH BP with only a minute peak cochromatographing with the 9,10-diol of BP.

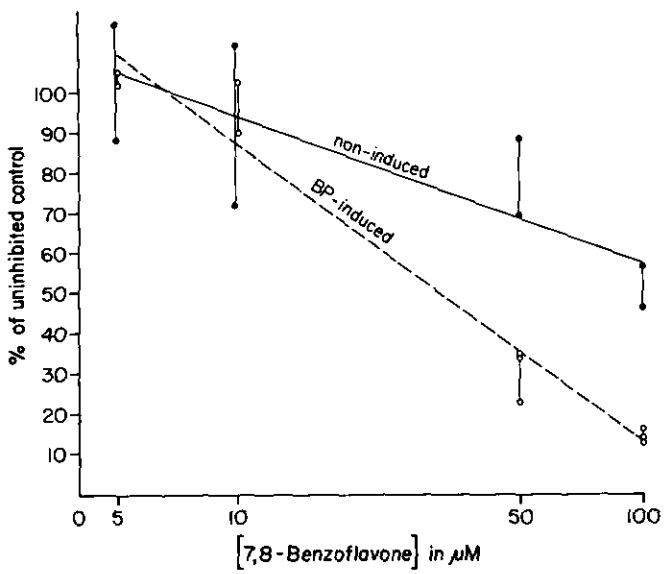

FIGURE 2. *Effect of* 7,8-BF *on benzopyrene monooxygenase activity* in supernatants from 10,000*g* centrifugation of hamster tracheal epithelial cell homogenates. Incubation time was 5 min at 37°C; concentration of [^{3}H]BP was 5 μM.

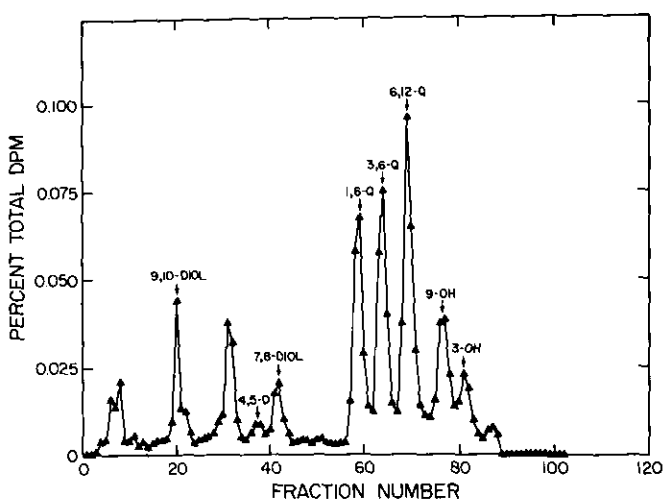

FIGURE 3. Metabolites produced by microsomes from BP-pretreated hamster tracheas after 30 min incubation with [^{3}H]BP and cofactors at 37°C. Separation by reverse-phase HPLC.

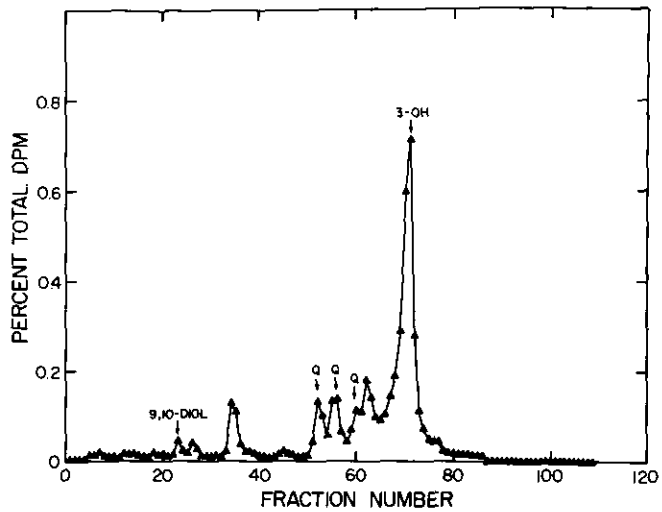

FIGURE 4. Reverse-phase HPLC separation of metabolites produced by rat tracheal microsomes isolated from BP-pretreated tracheas. Microsomes were incubated for 30 min with [^{3}H]BP and cofactors at 37°C.

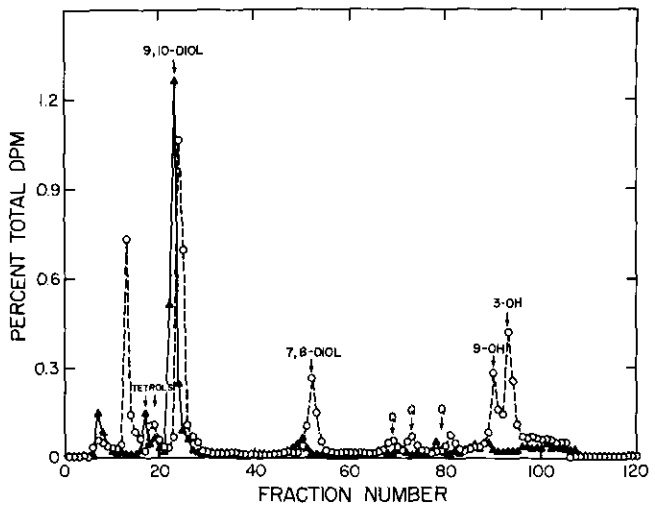

FIGURE 5. Hamster tracheas were incubated with 1 μM [^{3}H]BP for 24 hr and variously treated organ culture medium: (▲) extracted with ethyl acetate/acetone and organic solvent soluble metabolites separated by reverse-phase HPLC; (○) exposed to 11 units of aryl sulfatase and 1000 units of β-glucuronidase at pH 5 for 2 hr prior to extraction with ethyl acetate/acetone and separation by HPLC.

To determine whether the observed differences between rat and hamster in the activity microsomes and the distribution of products formed were reflected by the metabolism in intact tracheal cells, further studies of metabolism were done with tracheas in organ culture. After rat and hamster tracheas had been incubated with [^{3}H]BP, a portion of the medium was extracted with ethyl acetate/acetone and the remainder was treated with β-glucuronidase and aryl sulfatase prior to extraction. By comparing the metabolites extracted in these two ways, it was possible to determine both the quantities and proportions of water-soluble metabolites released into the ethyl acetate/acetone-soluble phase by the deconjugating enzymes. About 35% of the metabolites released into the medium by organ cultures were ethyl acetate/acetone-soluble, and the remainder were water-soluble. Deconjugation by aryl sulfatase and β-glucuronidase released an additional 30% of the total products; nearly 40% of the metabolites were still water-soluble after the enzyme treatment. The metabolites which were conjugated were mostly BP-phenols, BP-quinones, 7,8-diol, and highly polar derivates, some of which were polyhydroxylated (Fig. 5). The quantity of 9,10-diol which was ethyl acetate/ acetone-extractable did not change after the enzyme treatment. Rat tracheal organ cultures produced less ethyl acetate/acetone- and water-soluble metabolites per millgram of tracheal tissue than did hamster tracheal organ cultures (a total of 3.7 pmole/mg/24 hr and 7.8 pmole/mg/24 hr for rat and hamster, respectively). The major ethyl acetate/acetone-extractable metabolite was the 9,10-diol (Fig. 6). BP-phenols and a peak which contains a monohydroxy BP sulfate conjugate (BP–SO_4) comprised the next most abundant group of metabolites. Treatment of the medium with aryl sulfatase and β-glucuronidase released an additional 35%, but 43% of the

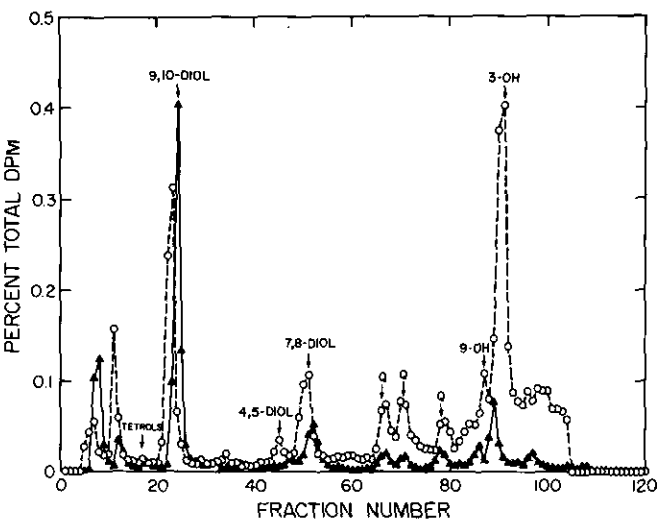

FIGURE 6. Rat tracheas were exposed to 1 μM [^{3}H]BP in organ culture medium for 24 hr: (▲) radioactivity from ethyl acetate/ acetone extract of medium separated by reverse-phase HPLC; (○) organ culture medium exposed to 11 and 1000 units of β-glucuronidase and aryl sulfatase, respectively, and metabolites extracted with ethyl acetate/acetone, and separated by reverse-phase HPLC.

radioactivity was still water-soluble. Enzyme treatment of medium from rat tracheal organ cultures released 3-OH BP as the major microsomal metabolite with lesser quantities of BP-quinones released. Virtually no BP-tetrols were present in the culture media (< 1.5 pmole), whereas hamster tracheas produced 15 pmole. The ratio of ethyl acetate/ acetone-soluble metabolites released from medium by aryl sulfatase and β-glu-

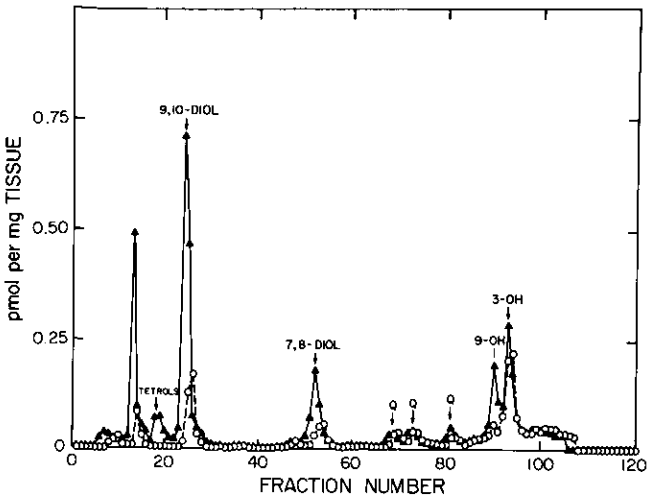

FIGURE 7. Comparison of metabolites produced by (▲) hamster tracheas or (○) rat tracheas after exposure of organ culture fluid to β-glucuronidase and aryl sulfatase. The results have been normalized for the weight of tissue utilized in the incubation.

Table 2. Comparison of metabolites produced by rat and hamster tracheal organ cultures.

Metabolite	Metabolite produced, pmole/mg tissue/24 hr		Ratio (hamster/rat)
	Hamster	Rat	
Pre-tetrols	0.86	0.18	4.78
Tetrols	0.22	0.01	22.0
9,10-Diol	1.41	0.30	4.70
4,5-Diol	0.05	0.04	1.26
7,8-Diol	0.46	0.15	3.07
Quinones	0.45	0.30	1.50
9-OH	0.48	0.11	4.36
3-OH	0.61	0.56	1.09

curonidase varied from 1.09 to 22.0 for hamster versus rat tracheas (Table 2). The greatest discrepancy in ratios is that of BP-tetrol formation; hamster tracheas produced 22-fold more BP-tetrols than did rat tracheas. This difference in products is further amplified in Figure 7.

In light of differences in metabolism noted for rat and hamster tracheas, studies of binding of BP to DNA of tracheas in culture were performed to further compare these two species. DNA was isolated from epithelial cells and banded on isopyknic CsCl gradients. The mean binding level for rat tracheas was 1.55×10^{-4} pmole bound/μg DNA. A separate experiment to determine the effect of a pretreatment with BP on binding levels revealed a binding level of 1.23×10^{-4} pmole/μg DNA in tracheas pretreated with BP whereas control tracheas had a level of 0.91×10^{-4} pmole/μg DNA. In hamster tracheas BP-pretreatment resulted in a greater than 2-fold increase in binding (16.9×10^{-4} versus 42.6×10^{-4} pmole/μg DNA) which is in substantial agreement with the earlier study by Kaufman et al. (*24*). The mean binding level for hamster tracheas was 26.7×10^{-4} pmole/μg DNA. The 17-fold greater binding to DNA in hamster tracheas than rat tracheas was comparable to the ratio of BP-tetrol formation in these two species and may explain the species specificity for BP-induced carcinogenesis in the tracheas of hamsters (*14,53*).

Carcinogen Metabolism and Binding in Human Respiratory Tissues

A series of recent experiments have extended the study of metabolism and the binding of the activated carcinogen to cellular DNA to human bronchial tissue. These studies were of obvious importance because the ultimate goal of previous studies, realized in these investigations, is knowledge of the metabolism of carcinogens in the critical human target tissue. Harris et al. (*27*) demonstrated that specimens of human bronchus could catalyze the binding of PNH to DNA of epithelial cells in explant culture of human bronchus. The specific activity of binding of 4 PNH [DMBA, BP, MCA and dibenzanthracene (DBA)] to bronchial cell DNA in two to four unique specimens was assessed and had a mean of 40 pmole/10 mg DNA; however, the specimens bound more DMBA and BP than MCA or DBA (*27*). In a study of the levels of binding of BP and DMBA assessed in 28 specimens of human bronchus, the mean level for DMBA–DNA binding was 118 pmole/10 mg DNA and that of BP–DNA binding was 38 pmole/10 mg DNA, indicating that DMBA was bound to a significantly greater extent. The levels of binding of BP to bronchial DNA were studied more intensively in 37 specimens from patients with or without lung cancer (*25*). The specimens were obtained either after immediate autopsy or during surgery to ensure the viability of the tissues. The tissues were placed in culture for 7 days prior to the addition of [^{3}H]BP to the culture medium to reduce the contribution by any exogenous or endogenous factors which could influence the levels of BP–DNA binding (i.e., specimens from smokers, or those taking medications). All tissues were resected from normal segments of the bronchus when specimens from patients with lung cancer were used. The levels of binding of BP to DNA in these specimens of human bronchus varied by 75-fold. In these initial studies there was no obvious relationship between levels of binding in specimens from patients with or without lung cancer. A more recent report (*26*) using 79 specimens showed a 150-fold range of variation in BP–DNA binding. Interestingly, when specimens from lung cancer patients were segregated according to histologic type of the tumor, a difference was noted. Binding levels were higher than controls in specimens from patients with epidermoid carcinoma, epidermoid combined with adenocarcinoma, and in mucous differentiated cancers with a nonglandular pattern. The mean binding level in specimens from patients with glandular, mucous differentiated cancers was not significantly different from controls. The metabolism of both cyclic and acyclic *N*-nitrosamines was assessed in human bronchial organ cul-

tures and these compounds were found to be metabolized to products which bound to cellular DNA (*54*). The capacity of human bronchus to activate aflatoxin B_1 to derivatives which bind to DNA also was determined; the values obtained were somewhat lower than with BP (*55*).

The range of variation in levels of carcinogen binding to human bronchus was similar to those observed with other human tissues. There was a 99-fold variation in the binding of BP to DNA in cultured human esophagus (*56*), 100-fold in colon (*57*) and 70-fold in human endometrium (*58*). The absolute values for the binding of BP to DNA in human tissues were highest in human bronchus and esophagus, lower in endometrium, whereas human colon bound approximately 1/10 the quantity of BP to DNA than did bronchus. The mean binding level for human bronchus (25 pmole/10 mg DNA) was close to that seen for tracheas of Syrian golden hamsters in culture (*14*). The factors which govern the levels and variation in BP–DNA binding levels in human bronchus have not been determined. The levels of binding were induced by BA pretreatment and by BP itself, and the binding was time- and temperature-dependent (*15*). The variation was apparently not related to the formation of different BP–DNA adducts, since the major adduct in bronchus, esophagus and colon is an adduct of (±) 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene with guanine (*28,56,57,59*). A likely determinant of the levels of binding of BP to DNA is variations in levels and species of cytochrome P-450. It has been observed that the total metabolic capacity for BP in microsomes from human lungs vary (*60*), and Genta (unpublished observations) (Fig. 8) has noted that 7,8-BF inhibits the binding of BP to DNA by 40% to 80% in bronchial specimens from nine individuals. Compounds other than 7,8-BF (*15*), including butylated hydroxytoluene (BHT) and nicotine, have been tested for their effect on BP–DNA binding in human bronchial organ cultures. The antioxidant BHT is a potent inhibitor of binding and reduced the level of BP–DNA binding by 80% at a concentration of 2 μM. Nicotine, although effective as an inhibitor of AHH in lung homogenates, did not alter the level of binding in organ cultures of human bronchus (*15*).

The metabolism of BP has been characterized by HPLC in organ cultures of human bronchus. As with rodent respiratory tissues, human bronchus produced organic solvent-extractable and water-soluble metabolites of BP (*20,28*). The ethyl acetate/acetone-soluble components consisted of the 9,10-diol of BP and the more polar triols and tetrols. Water-soluble metabolites consisted of sulfate, glucuronide, and glutathione conjugates of BP metabolites including BP-tetrols, the 9,10-diol, 4,5-diol, 7,8-diol, BP-quinones and BP-phenols (*28*). Approximately half of the BP metabolites were water-soluble, and half can be extracted with ethyl acetate/acetone. When specimens from patients with or without lung cancer were analyzed with respect to BP-metabolite profiles, they were indistinguishable from those from controls (*20*).

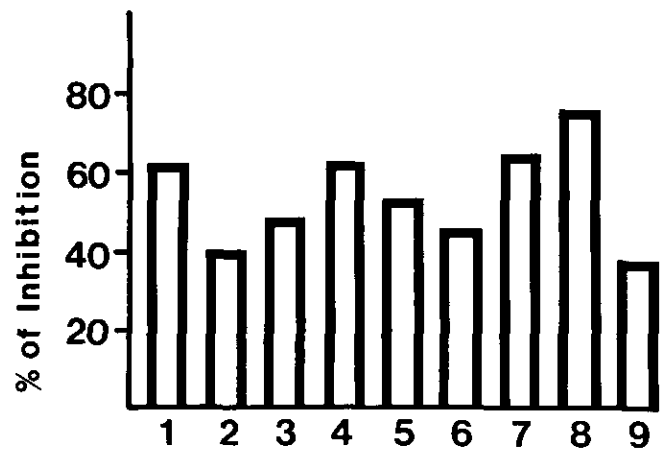

FIGURE 8. Inhibition by 7,8-BF of binding [^{3}H]BP to DNA in organ cultures of human bronchial epithelium from nine separate specimens. Ordinate, ratio of specific activity of BP binding to epithelial cell DNA in cultures with 7,8-BF added, to specific activity determined in control cultures × 100; abscissa, case identification number. Concentration of 7,8-BF and [^{3}H]BP were 2 μM; incubation period was 24 hr. Data of V. M. Genta (unpublished observations).

Effects of Retinoids on Tracheobronchial Epithelium

A second area concerned with the biology and biochemistry of tracheobronchial epithelium has been the subject of extensive study and progress in recent years. This is the various effects of retinoids on this tissue. The term "retinoids" has been used to describe the natural forms of vitamin A and the numerous functional and structural analogs of vitamin A that have been synthesized. Studies of retinoids have concerned biochemical and biological studies of retinoids, and investigations concerning their metabolism and mechanisms of action.

Relationship of Retinoids to Lung Cancer

The interest in the effects of retinoids on tracheobronchial epithelium stems from the observations of Saffiotti et al. (*61*) that administration of large doses of vitamin A to hamsters previously treated intratracheally with BP–ferric oxide caused a marked reduction in the number of respiratory tract tumors developing in this animals. Concurrent studies with natural and subsequent studies with natural and artificial retinoids have shown administration of high doses of retinoids to exert an inhibitory or preventive effect on carcinogenesis in several other organs (*38*). The subsequent record of studies of the effects of retinoid treatment on respiratory carcinogenesis has been less consistent. Retinoids

have been shown to inhibit respiratory carcinogeneis (*61–64*) or to have no effect (*65–67*) or even to potentiate respiratory carcinogenesis (*68*). The general interest in retinoids generated by these studies led to consideration of the effects of the vitamin A-deficient state with regard to carcinogenesis. Genta et al. (*69*) explored the effects of vitamin A deficiency on the binding of BP to DNA in tracheas from vitamin A-deficient and normal hamsters. Their observation, that vitamin A deficiency enhanced the binding of BP to DNA, led to further consideration of the effects of the vitamin A-deficient state on carcinogenesis in this tissue. Nettesheim et al. (*41,62*) evaluated this issue in rats maintained in marginal, near-deficient vitamin A status. In comparison to rats provided a roughly normal vitamin A intake, after intratracheal instillations of MCA, the rats nearly deficient in vitamin A developed a greatly increased incidence of squamous nodules and of squamous carcinomas of the peripheral lung. This observation has been supported by three epidemiologic studies which suggest a relationship between vitamin A status and lung cancer risk. Bjelke (*70*) conducted a prospective study in which vitamin A intake levels were estimated from dietary history surveys in a large Norwegian population and found that lung cancer incidence was inversely correlated with vitamin A intake. Mettlin et al. (*71*) estimated vitamin A levels from vegetable consumption surveys in a retrospective study on a separate population. Again, a low estimated vitamin A intake was associated with a higher relative risk for lung cancers among subjects who smoked cigarettes. In a recent prospective study (*72*) serum retinol levels were determined in a large geographically defined population. Low serum retinol levels were found among subjects who subsequently developed an increased incidence of cancers including lung cancer. This correlation was also consistent for the development of epithelial cancers of various cell types in several tissues with a high incidence of cancer.

RNA Metabolism and Inhibitors of RNA Synthesis

Efforts have been made to determine the mechanism of action of vitamin A in tracheal epithelium and to elucidate the relationship between vitamin A and carcinogenesis. On the macromolecular level, it was hypothesized (*9,24*) that the morphologic manifestations of vitamin A deficiency might be detectable as changes in the types and classes of proteins synthesized and that this might be distinguished in the quantity and species of their RNA precursors. To test this, tracheas were excised from vitamin A-deficient or normal hamsters and were incubated in organ culture medium containing [^{3}H]uridine. Epithelial cells were scraped from tracheas by the method of Smith et al. (*8*), whole cell RNA and DNA was isolated by phenol extraction, and nucleic acids were applied to agarose–acrylamide gels. In comparison to RNA species from normal tracheas, the profile of RNA resulting from incorporated [^{3}H]uridine in gels from tracheal epithelial cells of vitamin A-deficient hamsters consistently had reduced levels of certain high molecular weight RNA species (Fig. 9). This difference in gel profiles could be reversed by treatment of vitamin A-deficient animals with retinyl acetate, restoring the proportion of high molecular weight RNA species.

To determine if vitamin A deficiency affected the

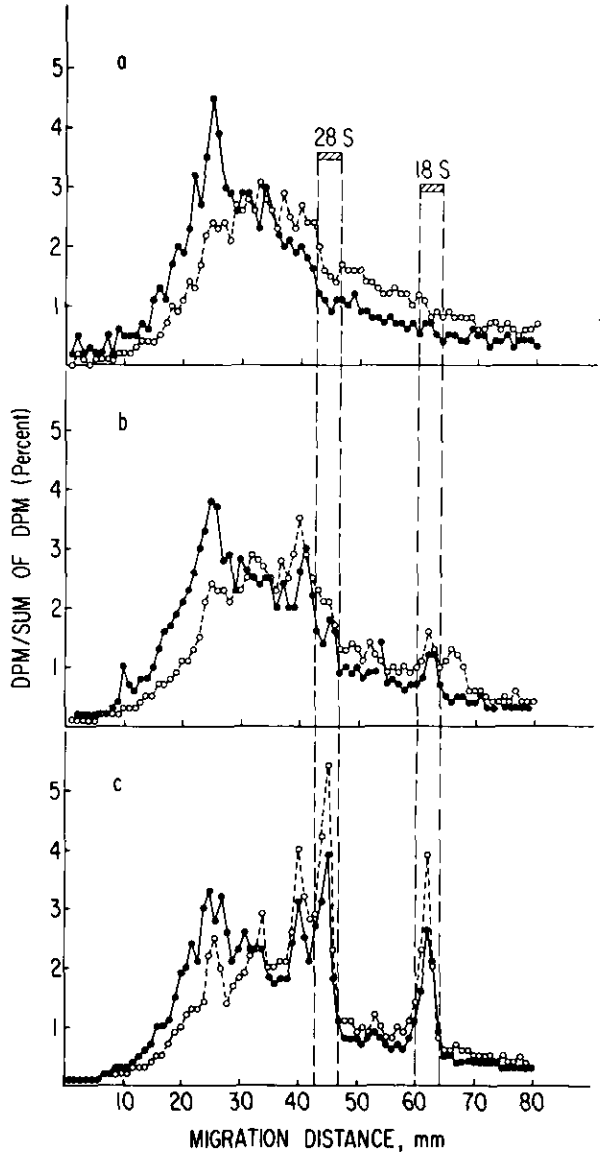

FIGURE 9. Electrophoretic profile of species of high molecular weight RNA synthesized *in vitro* in tracheal epithelium of normal and vitamin A-deficient hamsters. Distributions after labeling with [^{3}H]-uridine: (*a*) 30-min pulse; (*b*) 30-min pulse with additional incubation for 30 min; (*c*) 30-min pulse with additional incubation for 90 min; (○) RNA from tracheal epithelium vitamin A-deficient hamsters; (●) RNA from tracheal epithelium of vitamin A-normal hamsters. From Kaufman et al. (*88*) with permission of the authors and the American Association for the Advancement of Science.

processing or maturation of RNA, the effect of three inhibitors of specific steps in RNA synthesis were evaluated in organ culture. Toyocamycin, which inhibits the maturation of 18S and 28S ribosomal RNA species from their 45S precursor, actinomycin D and α-amanitin, inhibitors of RNA transcription and RNA polymerase II, respectively, were used to further characterize the effect of vitamin A deficiency. These compounds affected the RNA profiles in a manner consistent with their respective modes of action as described in other systems (*73*), therefore excluding a specific role for vitamin A in ribosomal RNA synthesis or processing. It was concluded that the effect of vitamin A deficiency was predominantly at the level of the heterogeneous nuclear RNA; these species of RNA are presumed to relate to messenger RNA. Since vitamin A deficiency is manifested as a change in the state of differentiation of this tissue, from a mucus producing to a squamous epithelium, it is plausible that an effect on messenger RNA could underlie or reflect the vast phenotypic changes seen in these cells.

Glycoprotein Biosynthesis

A major histologic change in respiratory epithelium of rodents deficient in vitamin A is a loss of secreting cells (*74*) whose secretory product is mucus, a glycoprotein mixture. Consequently, it was important to determine whether glycoprotein biosynthesis is altered in tracheas of vitamin A-deficient animals. Using vitamin A-deficient hamsters and rats, Bonanni et al. (*10,11*) showed that the amount of a glycopeptide containing fucose, mannose, hexosamines, galactose, and sialic acids synthesized by the rodent tracheas in cultures was dependent on vitamin A. In vitamin A-deficient rat tracheas the level of synthesis was a third of that in normal tracheas. More intensive studies of the mechanism of action of vitamin A in tissues such as liver (*75*), which allow more sophisticated analyses, indicate that vitamin A can act as a carrier in glycosyl transfer reactions in membranes. Conversion of vitamin A to retinyl phosphate apparently enables it to interact with GDP-hexoses and link with the sugar moiety, releasing GDP. The suger-charged retinyl phosphate can transfer sugar moieties to membrane-associated glycoproteins. This mechanism for the mode of action of vitamin A could explain the loss or reduction of glycoprotein species in vitamin A deficiency (*7,10,11,76,77*) as the result of a decreased capacity to manufacture mucus glycoproteins with specific sugar moieties. Further support for a role for vitamin A in glucoprotein synthesis came from work by Clark and Marchok (*76,77*). They reported that the type of mucins secreted by vitamin A-deficient rat tracheas in culture had an altered serine/glucosamine ratio.

Tracheal Organ Culture for Bioassay of Retinoids

Because of the interest in retinoids as possible cancer chemopreventive agents, it was necessary to develop a means for determining the potency and toxicity of retinoids in a relevant tissue. The ability of vitamin A to reverse morphologic alterations in vitamin A-deficient tracheal epithelium within days of administration was used by Sporn et al. (*35,36,38–40,78*) as an assay for retinoid potency. Tracheas excised from animals deprived of vitamin A and maintained in a serum-free, defined medium for 3 days were suitable for evaluating the effects of retinoids added to the medium. If an active retinoid such as retinoic acid were added to the medium at levels as low as 10^{-11}M, the squamous metaplasia and keratohyaline granules which indicated vitamin A deficiency diminished within 2 weeks, and normal morphology was restored. A quantitative estimate of efficacy was achieved by scoring a number of replicate cultures for the percent of cultures in which squamous metaplasia was reversed (Fig. 10). All-*trans*-retinoic acid was the most potent natural derivative of over 80 retinoids tested (*36*). Its 50% effective dose was 3×10^{-11}M (*36*). Tracheal epithelium in organ culture was approximately 10,000 times more sensitive to retinoic acid than were fibroblasts in cell culture (*79*). This indicates the need to evaluate the efficacy of such specific agents in the tissue in which its activity is relevant. Numerous retinoids have been synthesized and evaluated in order to identify compounds which are efficacious at low doses but are also free from toxicity. It appears as if the presence of the carboxylic acid, the alkene side chain, and the six-membered β-cyclohexenyl ring are all important determinants of the activity of retinoids; however, no simple

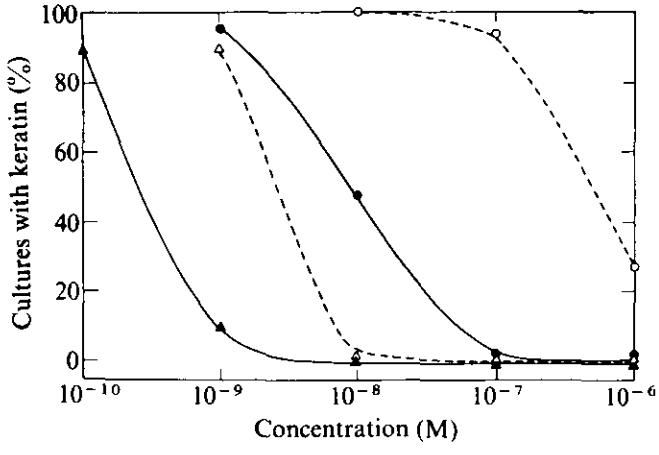

FIGURE 10. Bioassay for reversal of keratinization in tracheal epithelium in organ culture. Tracheas were incubated for 7 days in the presence of retinoids prior to scoring. Dose-response curves for: (▲), retinoic acid; (Δ), retinyl acetate; (●), *N*-acetyl retinylamine; (○), *N*-retinyl phthalimide. From Sporn et al. (*40*) with permission of the authors and MacMillan Journals, Ltd.

structure activity relationship is presently recognized. Single substitutions in any of these regions can result in a molecule with a markedly lowered biological activity. Conversely, there now exist a number of synthetic retinoids that are modified on both the ring and side chain, yet display a potency greater or equal to that of retinoic acid (*36*).

Metabolism of Retinoic Acid

The work of Sporn and collaborators (*34,35,37,80*) has elucidated another important aspect of the action of retinoic acid: that retinoic acid, like many xenobiotics and lipid-soluble compounds, can be metabolized in the target tissue. For retinoic acid, this biotransformation is linked to cytochrome P-450 containing mixed-function oxidase since the metabolism is sensitive to α-naphthoflavone (7,8-BF), is NADPH-dependent, is inhibited by carbon monoxide and it is inducible (*37*). It has previously been shown that retinoids act as competitive inhibitors of the mixed-function oxidase in lung microsomal incubations using BP as the substrate (*81*).

Like many substrates for the mixed-function oxidase, retinoids can act as inducers for their own metabolism by this enzyme complex. The magnitude of induction is dependent upon the particular retinoid, and the extent of induction varies from tissue to tissue. Interestingly, the classical cytochrome P-450 and P-448 inducers, phenobarbital and MCA, are ineffective as inducers of retinoic acid metabolism.

Roberts et al. (*37*) assayed for retinoid-induced metabolism of retinoic acid and derivatives *in vivo* and *in vitro* using hamster kidney, liver, trachea, testis and intestine. Vitamin A-deficient hamsters were given retinoids orally for induction for 3 days, after which [^{3}H]retinoic acid was injected intrajugularly and tissue excised after 4 hr. The metabolism of retinoic acid was found to be inducible *in vivo* in liver, kidney, and intestine. Both the testis and trachea had a high metabolic capacity for retinoic acid; however, the activity was independent of induction (i.e., was not inducible). Vitamin A-normal animals were much more metabolically competent at retinoic acid metabolism than the deficient animals, indicating that some degree of induction is present in the normal nutritional state.

The metabolism of retinoic acid has been characterized in detail in hamster tracheal organ cultures by Frolik et al. (*34,35*). Two major metabolites were detected after separation on reverse-phase HPLC. Derivativization and subsequent mass spectral analysis indicate that one metabolite is 4-hydroxyretinoic acid and the other is 4-oxoretinoic acid. When assayed in tracheal organ culture, both of these compounds exhibit little biological activity, and are on the order of 3% as active in reversing keratinization as is retinoic acid. Therefore, these metabolites were not considered "active intermediates" responsible for the activity of retinoids, and are likely to be products of inactivating pathways for retinoids (*34,35*).

Conclusion

The preceding discussion has focused on two areas of progress in studies of the biochemistry of tracheobronchial epithelium. As noted earlier, these topics were the subjects of investigations because biological studies of carcinogenesis in the rodent respiratory tract had revealed that carcinogen metabolism and effects of retinoids were important contributing factors. Within the framework provided by the biological observation of carcinogenesis studies, biochemical studies in both areas could proceed in an effort to relate biochemical mechanism to biological observation. Studies of carcinogen metabolism appear to have provided a mechanistic explanation for the vast difference between two species in susceptibility to tracheobronchial carcinogenesis by BP (*13,14*). They also may be providing an explanation for the specific risk of certain individuals to lung cancer. Furthermore, other studies in tracheobronchial epithelium have provided initial or important contributions to knowledge regarding the metabolism of BP-quinones (*21*), the mechanism of action of vitamin A (*82,83*), and the alterations in RNA metabolism related to reprogramming of cells for an altered state of differentiation. Several of these lines of investigation have proceeded with technical methods that were highly sophisticated and comparable to those used with other cells or tissues where quantities of material were far less of a problem.

In addition to providing a record of progress, these studies also illustrate a field of opportunity. Although studies with tracheobronchial epithelium still may be difficult because of the time and expense of obtaining the tissue, once the tissue is available, a wide range of biochemical investigations can be performed. Where a specific problem must be evaluated directly in this tissue, the methods exist for many types of studies and the opportunity exists for further development. Initial efforts were made based upon observations in respiratory carcinogenesis studies but the approach taken to study the biochemical features of carcinogenesis models can be applied to investigations of the pathogenesis of other diseases in this tissue.

With the development of techniques for the establishment of primary cell cultures and lines of bronchial and tracheal epithelial cells (*29–32,84–87*), the opportunities for the study of the biochemistry of the tracheobronchial epithelium may by greatly expanded. In addition to the reduction in cost resulting from the use of cultured cells rather than large numbers of animals, the obtainment of cells is much easier and the cell populations are far more homogeneous than those collected from animals. Furthermore, the availability of cell lines at various stages in the progression from normal to malignant may provide exciting opportunities for study

of the critical biochemical changes in premalignant and malignant tracheobronchial epithelial cells.

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Heterotopic Tracheal Transplants: Techniques and Applications

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Heterotopic tracheal transplants, placed subcutaneously in syngeneic rats have been extensively used in our laboratory. The objective of these experiments was to study the toxic and/or carcinogenic effect of several compounds on the respiratory tract mucosa. This was attained by exposing the transplants to an intraluminal pettet containing the toxicant or carcinogen mixed with an adequate matrix (gelatin, beeswax, stearyl alcohol, silastic, etc.). By varying the concentration of the test chemicals, it is possible to study dose-response relationships, and by changing the pellet matrix, the effects of release rate (dose rate) can be analyzed. Several end points can be studied, such as histological changes in the mucociliary epithelium, changes in mucus secretion, tumor induction and changes in the *in vitro* behavior of the epithelial cells after *in vivo* exposure. In addition, by de-epithelializing the tracheal transplants and reseeding them with another cell population, e.g., from previously treated cell cultures or from human specimens and transplanting them subcutaneously in nude mice, completely new vistas on the effect of chemicals can be opened.

Introduction

The direct application of toxicants and/or carcinogens to the respiratory tract epithelium by inhalation exposure, intratracheal injections or pellet implantation into the bronchus or lung has been frequently employed in toxicology and carcinogenesis studies (*1–3*). Although each of these techniques offers some advantages, with the first two it is often difficult to determine the precise dose delivered to each segment of exposed airway mucosa. Furthermore, multiple exposures are usually required to obtain the desired effect, and several portions of the respiratory tract may be exposed to different doses of the test substance. The pellet implantation technique obviates these problems by exposing a circumscribed area of respiratory mucosa to a known quantity of test substance which diffuses out of the pellet, either into the bronchus or into the lung. This technique, on the other hand, is complicated and often impairs the normal respiratory function of the treated lung, not infrequently leading to premature death of the animals. A model, which has the advantages of the *in loco* pellet implantation technique and does not jeopardize the respiratory functions of the experimental animal, was developed in our laboratory by transplanting subcutaneously tracheas obtained from syngeneic rats (*4,5*). Previous experiments by other investigators had shown that lung parenchyma as well as tracheas could be transplanted and exposed to carcinogens (*6–9*). Some of these early studies were successful in producing carcinomas after methylcholanthrene or dibenzanthracene exposure, and papillomas after diethylnitrosamine exposure. The approach used in our laboratory is to transplant tracheas subcutaneously and expose them only after the grafts have been fully established and revascularized. The use of several types of pellet-matrices has permitted the controlled release of varied total doses of test substances at a predetermined dose rate. The combined use of fully established and revascularized transplants and slow release pellet matrices has eliminated to a great extent, hyperacute toxic damage to the graft and surrounding tissues, as well as decreased the incidence of peritracheal sarcomas, which have been previously reported (*9*).

Techniques

Since most of our own work, as well as the majority of the experiments published to date, has been carried out in rats, the following description of the transplantation techniques will deal mainly with the procedures using this species. Differences or variations in techniques when using other species will be mentioned when appropriate.

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Donor Tracheas

Ten- to twelve-week-old inbred specific pathogen-free Fischer 344 rats are used as donors for tracheas. A wide anterior thoracotomy is performed under anaesthesia with either methoxyfluorane or sodium pentobarbital (Fig. 1). After exposure of the anterior mediastinum under strict aseptic conditions, the trachea is separated from the surrounding tissues, and a polyethylene tubing (length 20 mm, outer diameter 5 mm) is sutured to one side of the trachea to preserve the original length of the organ. If this step is omitted, the trachea contracts to approximately one-half of its original length and tends to curl. The trachea is severed below the first tracheal ring and immediately above the carina. The laryngeal end of the trachea is tied up with a silk suture. The trachea is removed from the donor animal and placed in a Petri dish containing Hank's balanced salt solution with streptomycin and penicillin. The tracheas can be kept in this solution for several hours without any apparent loss in viability. Before transplantation, the other extreme of the trachea is closed with a silk suture. This step can be omitted without noticeable differences because after transplantation the open end will be closed by granulation tissue.

Recipient Animals

Rats of the same strain and age are used as recipients. They are anesthetized with methoxyfluorane, their backs are shaved with electric clippers and cleansed with 5% peracetic acid solution in water, and an incision, 1 to 2 cm long near the midline, is made on the dorsum, parallel to the vertebral column between the scapulae. With blunt dissection, two subcutaneous pouches, one at each side of the incision, are carefully prepared to lodge the tracheal transplants, together with their supporting polyethylene tubing (Fig. 2). In order to standardize the position of the tracheal transplant (tt), the transplant is so placed that the distal end points toward the head of the recipient animal. The wound is then closed with surgical clips and the animal with one tracheal transplant at each side of the dorsal midline is returned to its cage. For practical purposes, usually no more than two tracheas are transplanted per animal, but in some cases, more transplants per animal can be used with little inconvenience (*10*). This is not advisable if during a long-term study the tracheal transplants are likely to develop either tumors or extensive fibrosis, situations which interfere with the usually nonsynchronous processes taking place in the neighboring tracheas.

Similar techniques of syngeneic tracheal transplants have also been applied with another rat strain (*10*) and other species, i.e., hamsters (*5,11,12*), dogs (*13–15*), and mice (*5,16*). After transplantation, the tracheal transplants become revascularized in approximately 5 days. During the first 2 days, the respiratory epithelium becomes necrobiotic, and most cells slough off (*5*). Remaining basal cells rapidly repopulate the epithelium, which is completely reconstituted and normal after 2

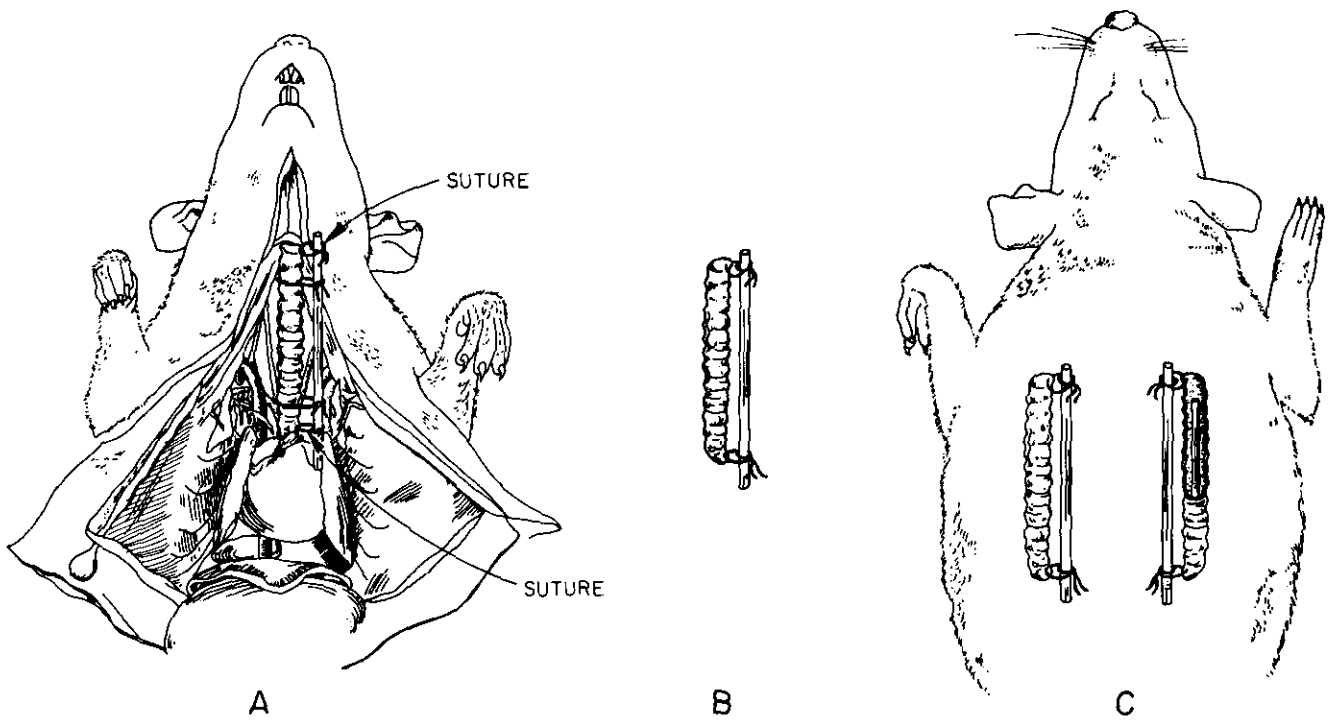

FIGURE 1. Transplantation procedure: (*A*) the wall of the donor trachea is sutured to a polyethylene tubing (arrows), and both ends are tied up by another pair of sutures; (*B*) the trachea and the tubing are excised and (*C*) transplanted in the dorsal subcutaneous tissue of a syngeneic recipient.

FIGURE 2. Tracheal transplant in the subcutaneous tissue of the rat dorsum. The skin has been resected for better view.

weeks. Between 2 and 4 weeks after transplantation, the tracheal transplant is completely revascularized, and the epithelial lining becomes indistinguishable from that of the host trachea (Fig. 3). It is usually at this time that the tracheal transplant is exposed to chemical agents. The transplant is exposed to carcinogens or toxicants in a controlled and quantitative fashion by inserting the appropriate pellets (see below), containing the test chemical, into the tracheal lumen. This is achieved through a small incision, which can be easily closed by either a simple suture or by using large tantalum hemostatic clips (E. Weck Co.). Likewise, if the exposure is to be terminated before the tracheal transplants are removed, the pellets are extracted through another incision at the cephalic end of the transplant and closed again.

Materials and Vehicles for Intratracheal Pellets

The selection of the pellet vehicles or matrices depends on the nature of the materials to be released from the pellet and on the desired rate of release. In most cases, a long exposure of the tracheal mucosa to constant concentrations of chemicals and in consequence a slow release of the test chemical from the pellet is desired. Tracheal transplants can be exposed to two types of test agents which require different vehicles and pellet manufacturing techniques: lipophilic substances, such as polycyclic aromatic hydrocarbons (PAH) and 12-O-tetradecanoyl-phorbol 13-acetate (TPA), which are incorporated into nondegradable matrices such as beeswax (*17*), silastic (*18*) or lycra fibers (*12*) and poorly soluble particles or fibers, such as nickel subsulfide, asbestos or arsenic trioxide embedded into degradable vehicles such as gelatin. This latter approach implies relatively short exposure times and an abrupt release of the test agent since gelatin dissolves rapidly at body temperature. Alternative matrices for poorly soluble particles and fibers which enable longer exposure times are under development.

Technology of Pellet Production

The rate of release of the test agent from the pellets depends mainly on the nature of the pellet matrix. Materials for pellet matrices must meet the following criteria: should be nontoxic; should not interact with the carcinogen/toxicant; should be easily fabricated into solid cylindrical pellets (14 mm × 1.4 mm diameter for rat trachea) at low temperatures to avoid any decomposition of the test chemical. Release from such monolithic cylindrical pellets containing the dispersed test agent is controlled by the rate of diffusion of the chemical through the pellet matrix to the boundary surface as proposed by Higuchi (*19*). Invariably, an initial high release rate (burst effect) occurs (*20*) until the equilibrium is reached and the release takes place in a controlled fashion.

Lipid Matrices. Beeswax, stearyl alcohol and cholesterol have been employed in our laboratory for slow release of PAH and TPA. Relatively low-melting (up to 150°C) matrices containing the dissolved/dispersed

FIGURE 3. Normal mucociliary epithelium lining the tracheal lumen 7 weeks after transplantation. (Toluidine blue, Epon, × 360.)

agent can be fabricated most conveniently in the pellet maker (*4*) (Fig. 4). The test agent and lipid substance is put into a glass scintillation vial and placed in the recess of an aluminum block which is heated on a thermostatically controlled electric heater. The block should be drilled for accommodating a thermometer to monitor the temperature. Heaters (Cole Palmer Instruments) equipped with immersion thermistor probes have been found satisfactory. These probes are placed in another cavity drilled in the block. Occasionally, it is difficult to push the pellets out of the mold by using the push pin. In such a situation, the mold should be reheated by placing in a water bath to a temperature which facilitates extrusion.

The pellet maker works best if the dispersed test agent does not show any tendency to settle. If settling occurs, the matrix is melted in a hot mortar and the agent is mixed with the matrix thoroughly, using the pestle and mortar and allowing it to cool until it solidifies. The mix is scraped out from the mortar and packed into a cylindrical mold of a sodium press (No. 1 Greenerd Arbor Press, Fisher Scientific Co.). This mold is fitted with a die with a 1.5 mm diameter orifice, through which the pellets can be extruded by manually driving a plunger fitted with a rack and pinion mechanism. The mold is warmed to a suitable temperature (below the melting point of the mix) and extruded out quickly. The extruded material is cut into proper lengths by using a channeled Teflon block very much like a carpenter's miter box.

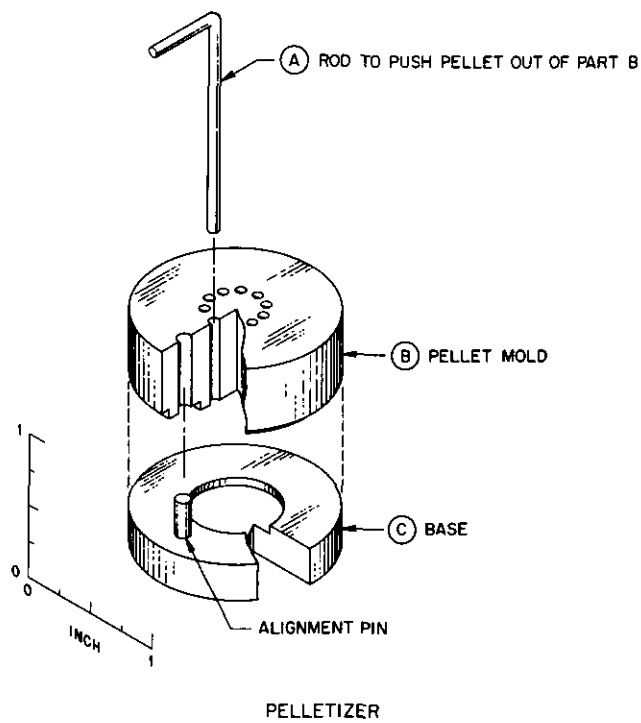

FIGURE 4. Schematic representation of the pellet maker used for manufacturing carcinogen-containing beeswax or gelatin pellets. From Griesemer (*4*).

Pellets Containing PAH. Controlled release of polycyclic hydrocarbons like benzo(a)pyrene (BaP), 7,12-dimethylbenz(a)anthracene (DMBA) from beeswax alone and from cholesterol–beeswax matrices has been reported earlier (*17*). The presence of cholesterol in a beeswax–cholesterol matrix retards the release of PAH in direct proportion to the percentage of cholesterol present. The use of cholesterol of highest purity is essential, and commercially available highest purity cholesterol (Eastman Kodak) should be purified further by the method of Fieser (*21*) and checked for purity by gas chromatography on a 3% SP-2250 column (Supelco).

We have recently found an undesirably high background in the epithelial focus (EF) assay (*22*, M. Terzaghi, unpublished data) as well as the induction of stratified metaplasias after exposing tracheal transplants to cholesterol. In addition, some cholesterol autoxidation products have been found to be mutagenic (*23*). Stearyl alcohol has been found to be an acceptable substitute for cholesterol since it shows very low background in the EF assay. The release rates of DMBA

from beeswax–stearyl alcohol (1:9) and beeswax–cholesterol (1:9) matrices are shown in Figure 5. The use of beeswax is necessary as a binder, since pellets are extremely fragile and difficult to handle when made out of cholesterol or stearyl alcohol alone. A typical preparation of stearyl alcohol–beeswax DMBA pellets is described below.

Weighed amounts of DMBA (Eastman Kodak), purified by crystallization from ethanol as described previously (*17*), and stearyl alcohol, and beeswax (purified by filtration through a steam-heated fritted glass funnel) are heated together in a scintillation vial placed in an aluminum block until a clear melt is formed (65°C). The hot melt is transferred into the well of the pellet maker heated to the same temperature and the matching half is placed on the well, forcing the melt to flow into the holes where, following rapid chilling, cylindrical pellets are formed. A copper block (7.5 cm × 7.5 cm diameter) chilled in ice was found to be very convenient for cooling the pellet maker.

In order to directly assay the amount of DMBA in the pellets, a weighed aliquot of the pellet is dissolved in 10 mL of benzene in a capped scintillation counting vial placed in a shaker water bath at 37°C. The absorbance at 301 nm is read after appropriate dilution, and the concentration of the DMBA determined using a molar extinction coefficient of 78,700. Neither beeswax nor stearyl alcohol or benzene shows any appreciable absorbance at this wavelength. The pellets are best stored in a deep-freeze under nitrogen in the absence of light.

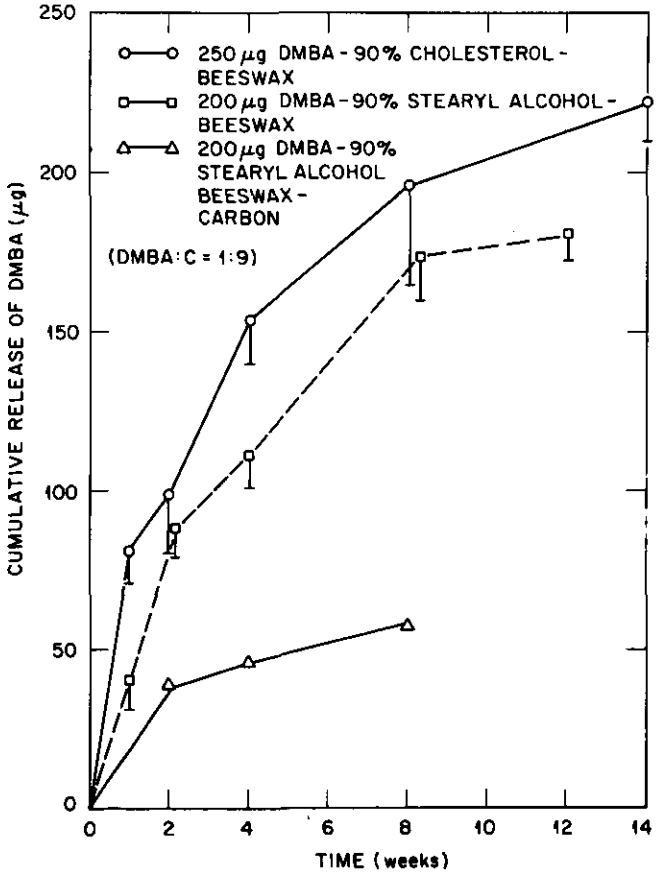

FIGURE 5. Release rates of DMBA from several pellet matrices.

If a slower release rate is desired, DMBA may be absorbed on carbon particles and dispersed in the stearyl alcohol–beeswax (9:1) matrix and made into pellets using the sodium press. Carbon particles (Barnaby Chaney) are passed through sieve #200 and sieve #325 and the particles retained on the latter are used.

DMBA–carbon (1:9) is prepared as follows. Carbon particles (9.0 g) are added with stirring to a hot solution of DMBA (1.1 g) in acetone (160 mL) in a beaker. The acetone is allowed to evaporate in the hood at room temperature. All of the dried, caked mixture from the wall of the beaker is scraped down, and the side of the beaker rinsed with 3 mL acetone. The mixture is heated gently with constant stirring and scraping. After all of the acetone has evaporated, the mixture is heated to about 145°C, with continued scraping and stirring. The mixture becomes dry and powdery. The final product is cooled in a desiccator. To assay the amount of DMBA in the mixture, samples are extracted in a double thickness cellulose thimble (Whatman 1317–403) placed in a Soxhlet apparatus with benzene. Ultraviolet absorption spectrum measurements show that DMBA is not decomposed by this treatment and the absorption of DMBA into carbon is uniform. Weighed amounts of DMBA–carbon are mixed together with stearyl alcohol and beeswax using a hot mortar and pestle, transferred into the mold, heated to 58°C, and extruded into pellets using the sodium press. The uniformity of the pellets can be checked by assaying for DMBA content. A typical *in vivo* release rate of carbon-adsorbed DMBA from these pellets is shown in Figure 5.

Pellets Containing 12–0-Tetradecanoylphorbol 13–Acetate (TPA). Beeswax can be used as a matrix and the TPA pellets can be conveniently made using the pellet maker. Since the molar extinction of TPA is low, it is advisable to use radioassay by incorporating [^{3}H]TPA for quantitative determinations of TPA release. An aliquot can be counted directly in a toluene-based scintillation fluid. Figure 6 shows the release pattern from a beeswax pellet containing 100 μg TPA.

Water-Soluble and Degradable Matrices. Twelve percent aqueous gelatin has been successfully used as a vehicle for rapid release of poorly soluble particles and fibers, such as Ni_3S_2, asbestos, carbon particles and glass fibers. It has also been used for the rapid release (and in consequence for short, acute exposures) of PAH.

Using a warm pestle and mortar, the agent is mixed thoroughly with the gelatin solution and allowed to cool to room temperature while triturating until the mix solidifies. It is then transferred to a scintillation counting vial and heated in an aluminum block to 30°C and

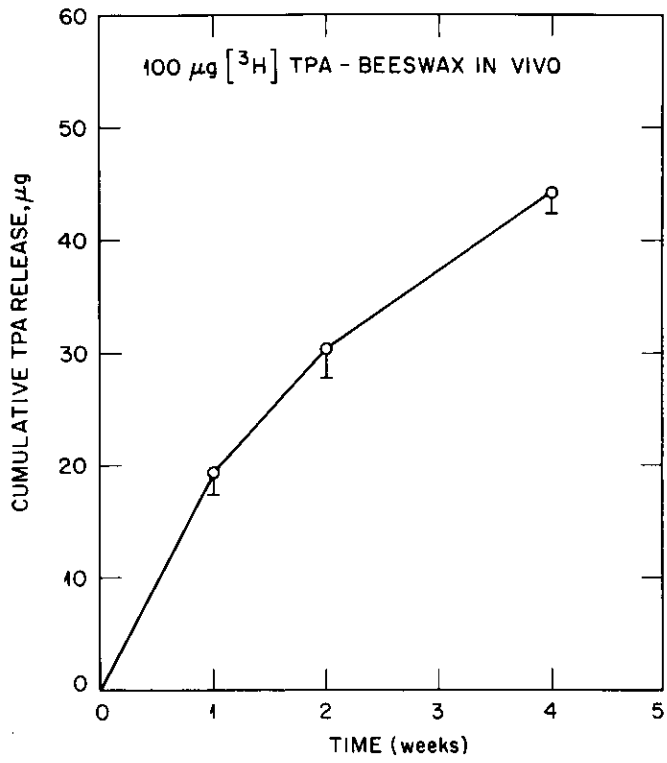

FIGURE 6. Release of TPA from beeswax pellets.

made into pellets using the pellet maker. The pellets are then dried on plastic Petri dishes placed inside a vacuum desiccator over sodium hydroxide pellets. The dried pellets are then assayed for uniformity. In most cases, the pellet is weighed, stirred in water, and filtered through a tared millipore filter. The filter is thoroughly washed with water, dried, and weighed. The concentration of agent in gelatin should not vary more than ± 5%.

Preliminary data suggest that particulate and fibrous material can be released in a continuous slow fashion from stearyl alcohol–beeswax (9:1 ratio), or polyglycolic acid pellets (material used in resorbable surgical sutures; in contact with body fluids it slowly degrades by hydrolysis). These matrices gradually release the embedded particulate material, which then comes in contact with the tracheal epithelium.

Evaluation of Effects

After a short- or long-term exposure to chemicals, simple macroscopic evaluation of the transplanted organ can reveal significant alterations, such as paratracheal or intratracheal cysts or aneurismal dilatations (frequently seen after destruction of part of the wall and subsequent migration of epithelium to an extraluminal position), solid tumors, atrophy and fibrosis, hypertrophy due to accumulation of secretion, etc.

Evaluation of Intratracheal Content. The type of intratracheal contents, as seen by naked eye, can point to the nature of the changes taking place in the epithelium, e.g., keratin can be recognized as semisolid white clumpy masses, and is indicative of squamous metaplasia and/or neoplasia, purulent secretion will signify inflammation and large quantities of mucous secretion will indicate hypertrophy of the secretory epithelium.

Biochemical analyses of the tracheal secretions have characterized the normal mucin fraction of the rat tracheal transplants (*24*). The purified mucin from the transplant contained high molecular weight glycoprotein with a carbohydrate and amino acid content characteristic of mucins. It is well known that similar fractions obtained from tracheal organ cultures undergo changes when exposed to an altered environment such as vitamin A-deficient media (*25*). Changes in the mucin secretion characteristics have also been described in neoplastic epithelial cell lines derived from carcinogen-treated tracheal epithelium (*26*). Mucous secretions from tracheal transplants could serve as an ideal model for biochemical studies of *in vivo* alterations after exposure to toxicants or carcinogens. The potential value of such a nondestructive analysis can not be overstated, and this model offers the possibility of performing long-term studies and obtaining multiple secretion samples from the same transplanted organs. Another type of nondestructive analysis, namely exfoliate cytology, can be performed easily in a longitudinal study of the effects of chemicals on the tracheal epithelium. Although this possibility has not been exploited, all the techniques are available (*27*) and the sampling procedures, which should produce minimum possible damage to the tracheal transplant, could be easily developed.

Histological Study of Tracheal Transplants. Histological observation of tissue sections is the most frequently employed method for evaluating the state of the epithelium and wall of the tracheal transplant. Most studies have relied on routine paraffin embedding, and some have preferred the higher resolution provided by plastic embedding (*28–31*). Several papers have dealt with the ultrastructural characteristics of the normal and altered epithelium (*28,29*).

The most critical issue when studying the distribution and frequency of lesions in the tracheal transplant is the sampling of each trachea. In most studies, the tracheas were cut transversally into rings 1 to 1.5 mm thick. The tracheal pieces are kept in proper order during dehydration and embedding by passing a silk thread through the surrounding connective tissue of each ring. The tissues were embedded maintaining the rings in proper order and orientation. Three to four sections 50 μm apart were then performed, thus further enlarging the sampled area. An alternative method, permitting an ample visualization of the luminal epithelium using only one or two blocks of tissue is

the so-called "Swiss roll," in which the trachea is cut frontally in two pieces, then rolled cephalocaudally, and tied with thread to keep it in this position (*32*). Another method which could permit a selective sampling of tracheal regions has been used for detecting squamous metaplasias of the larynx. This involves staining the whole organ (fixed or unfixed) in either pyronin (*33*) or alcian blue–phloxine (*34*) and then processing for histology only the positively stained areas. This method can be used for the detection of squamous metaplasias in the tracheal transplants, and it seems especially indicated when using tracheas from large animals.

In Vitro *Studies of* in Vivo-*Exposed Cell Populations.* *In vitro* techniques offer new possiblities for the study of sequential changes presumed to occur during the *in vivo* development of neoplasia. It has been shown that shortly after carcinogen exposure of tracheal transplants, epithelial cells which have a markedly increased growth capacity can be isolated from the tracheal mucosa (*22,35*). A series of techniques collectively referred to as the EF assay has been devised to quantitate *in vitro* the emergence of carcinogen-altered and neoplastic cell populations *in vivo* in tracheas exposed to carcinogen. A detailed protocol has been previously described (*22*). Briefly, the EF assay entails the following sequential phases. Heterotopic tracheal transplants are exposed to PAH as described above. At various time intervals after initiation of exposure, beginning prior to the development of frank neoplastic or even before the appearance of preneoplastic lesions, tracheal transplants are removed from host animals. Luminal epithelial cells are dispersed enzymatically and seeded into tissue culture dishes in order to establish primary cultures, the number of EF (proliferating epithelial cell foci) per dish is scored. When isolated, each EF contains roughly 5×10^4 cells. They are removed from the primary dish enzymatically and each EF seeded into a separate secondary culture dish. The fraction of EF which can be subcultured two or more times is noted. All subculturable EF (EF_s) are tested for the capacity to grow in soft agarose. In this system, the capacity to grow in soft agarose has been found to be well correlated with the capacity of a cell population to yield tumors when inoculated IM into immunosuppressed rats (*35,36*).

The EF assay has made it possible to monitor the development *in vivo* of cell compartments endowed with different proliferative and neoplastic potentials *in vitro*. Epithelia from normal noncarcinogen-exposed tracheas yield few EF in primary culture. Epithelia from carcinogen-exposed tracheas yield 10 to 100 times more EF than control cultures. The capacity of EF to be subcultured (EF_s) and the capacity of EF_s to grow in soft agarose (EF_s, ag+) appear to reflect the severity of carcinogen-induced changes in tracheal epithelium. The frequency of EF_s and EF_s, ag+ populations increase both with carcinogen dose and increased time after exposure (*22,37*).

Applications

Most studies employing the tracheal transplant model have dealt with the induction of neoplastic and preneoplastic lesions after *in vivo* exposure to polycyclic aromatic hydrocarbons. This model makes possible other studies in the field of toxicology and carcinogenesis. Some of the most recent applications are listed and briefly described below.

Early Effects of Toxicants and Carcinogens

The aim of these studies is to evaluate the toxic effects of several chemicals on the tracheal tissues (especially the epithelium) over an 8-week period. Most of the chemicals thus far evaluated in our laboratory were known or suspected carcinogens or closely related substances. Topping et al. (*38*) compared the acute effects of seven PAH on the tracheal epithelium by exposing the tracheal transplant with beeswax pellets containing 1 mg of pyrene (P), benzo(e)pyrene (BeP), anthracene (A), benz(a)anthracene (BaA), dibenz(ac)-anthracene (DBacA), benzo(a)pyrene (BaP) or dimethylbenz(a)anthracene (DMBA). The release of PAH from the pellets was determined by ultraviolet spectrophotometry at 3 days, 1, 2, 4 and 8 weeks. All PAHs were released continuously during the 8-week period. Approximately, 1 mg of PAH was released from the pellets containing P, BeP, BaP and A, whereas only approximately 500 μg was released after 8 weeks from the pellets containing the other PAHs. The toxic effects on the respiratory epithelium were evaluated by measuring the percentage of luminal surface occupied by normal, hyperplastic and metaplastic epithelia (Fig. 7). Although all compounds proved to be toxic for the tracheal epithelium, the degree of alterations induced in the tracheal transplants was variable, (i.e., the potent carcinogens (BaP and DMBA) showing a maximum of early toxic effects and the noncarcinogens or weak carcinogens (BeP, A, P) inducing less severe changes or short duration. A similar study was undertaken in order to evaluate the effects of chrysotile A and crocidolite asbestos in gelatin pellets (*39*). Both substances produced intense dose-dependent inflammatory reaction and epithelial alteration over a period of 4 weeks. The effect of crocidolite lasted somewhat longer than that of chrysotile A. Another carcinogen which induced extensive early toxic alterations was nickel subsulfide (*40*). Other particulates have been tested in the same model in our laboratory, i.e., arsenic trioxide, glass fibers, and charcoal particles. All these induced a weak or no response (Klein-Szanto et al., unpublished data). Although there are many complicated and interacting factors involved, (e.g., dose, dose rate, schedule of administration, etc.), these experiments indicate that strong carcinogens are usually very irritant and inflammatogenic for the respiratory tract epithelium, and that

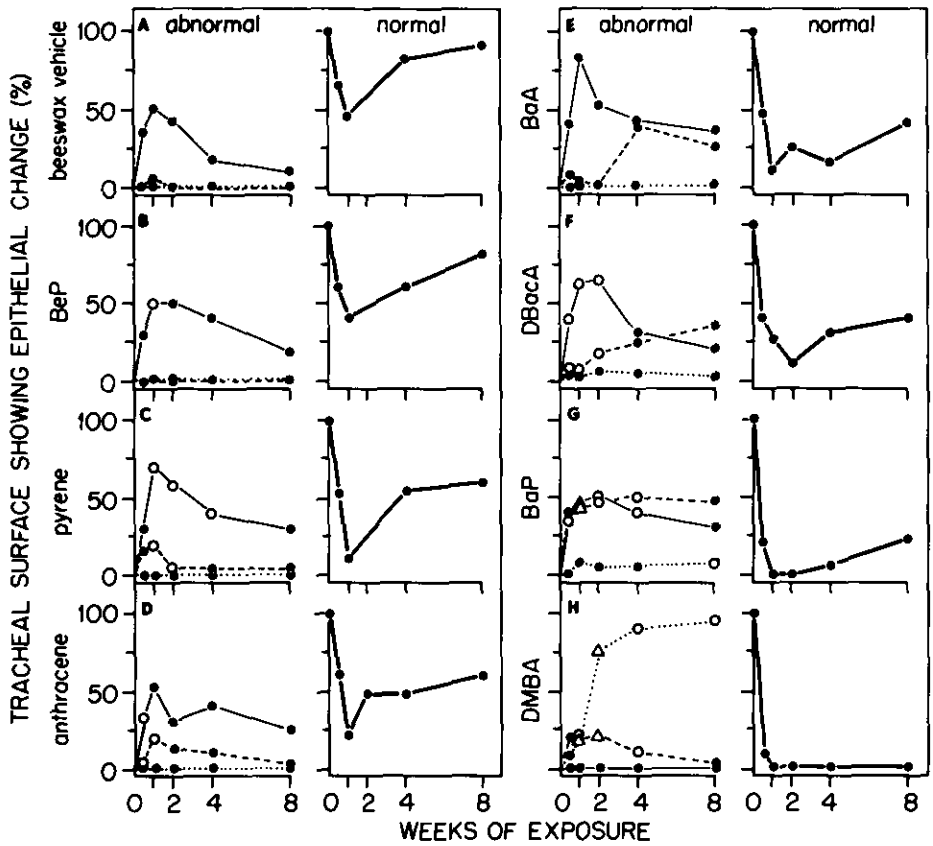

FIGURE 7. Extent and type of epithelial changes induced by various polycyclic hydrocarbons. The estimated percent tracheal surface area occupied by various epithelial morphologies is shown: (—) normal appearing epithelium; (—) hyperplasia; (--) transitional epithelium; (. . .) squamous metaplasia. Values are based on six tracheas per time point. Each trachea received 1 mg of PCH incorporated in beeswax. Control tracheas received pellets made of beeswax only. The intensity of the epithelial change is indicated as (●) mild; (○) moderate; (Δ) severe. From Topping et al. (*38*).

weak or noncarcinogenic related substances tend to induce a lesser reaction.

Tumor Induction and Cancer Morphogenesis

The advantage of the tracheal transplant model, i.e., knowing the precise quantity of carcinogen delivered to the tracheal mucosa, the exact period of time during which the mucosa is exposed to the carcinogenic agent, and the uniformity of exposure of the whole organ, have been exploited in several carcinogenesis studies. Using rat tracheal transplants continuously exposed to varying amounts of benzo(a)pyrene or 7,12-dimethylbenz(a)-anthracene in beeswax pellets, investigators in our laboratory have detected a clear dose-dependency in the tumor induction capacities of these PAHs (*41,42*) (Table 1). Although more than 90% of the carcinogen is released in 4 weeks from beeswax pellets containing less than 200 μg DMBA, a clear decrease in the number of induced tumors was observed when pellets were removed after 4 weeks of exposure (*43*). Only 20% of the tracheal transplants presented carcinomas, as opposed to approximately 60% when the DMBA containing pellet was left in for the whole duration of the experiment. These results are very similar to those obtained after a 4-week exposure to 200 μg DMBA-containing pellets, followed by a continuous exposure to 100 μg TPA. In this latter case, in which a two-stage carcinogenesis protocol, similar to the one employed in skin carcinogenesis studies (*44*) was employed, the percentage of induced neoplasm was 70 (*45*). From these experiments, it became obvious that the total time of exposure to PAH was at least as important as the total dose employed, and that a promotion-like effect elicited by either very small amounts of PAHs or by known promoters such as TPA or putative promoters like asbestos (*46*) is a probable pathogenic mechanism in respiratory carcinogenesis.

Using similar experimental conditions, we have extensively studied the morphogenesis of tracheal epithelial neoplasia, a detailed account of which is outside the scope of this chapter. The reader is referred to several recent reports on this subject (*43,47*). Briefly, the most interesting findings have been the reversibility of the early generalized toxic epithelial alterations induced by PAH (*47*) (Fig. 8), and the later appearance of focal

Table 1. Tumor development induced by BaP and DMBA in tracheal transplants.

PAH	PAH dose, μg	Total tumors/number of tracheal transplants
BaP[a]	45	0/10
	300	1/14
	900	2/12
	1250	7/13
	1740	15/10
	2160	18/12
	2490	40/52
DMBA[b]	10	0/24
	40	0/24
	115	7/24
	210	23/36
	325	11/11
	1240	12/12

[a] From Nettesheim et al. (*42*); includes all types of epithelial neoplasms.
[b] From Griesemer et al. (*41*); includes only carcinomas.

metaplastic–dysplastic lesions of varying degrees of atypia which usually precede the appearance of neoplasms (Fig. 9) (*43*). The number and degree of severity of these lesions also seems to be dose dependent. Of particular interest was the fact that many of the focal lesions regress after they reach a peak incidence, thus demonstrating that these lesions are not obligatory preneoplastic alterations.

Other studies have demonstrated a continuum of changes from the mildest to the more severe preneoplastic alterations. Quantitative techniques demonstrated increasing nucleus-cytoplasm ratios, ^{3}H-thymidine labeling indices and number of dark epithelial cells, indicating increasing degrees of cell proliferation and altered cellular maturation (*31*).

Other studies employing rat tracheal transplants have demonstrated the carcinogenic and promoting effects of asbestos (*39,46*), nickel subsulfide (*40*) and chromium carbonyl (*10*). The carcinogenic effects of PAH have also been studied in hamsters (*5,11,12*), mice (*5,16*) and dogs (*13–15*).

Repopulation of Tracheal Transplants with Isolated Cell Populations

It is often difficult to study the growth and differentiation of cultured or isolated normal or preneoplastic epithelial cells when transferred into an *in vivo* situation. Following subcutaneous or intramuscular inoculation they frequently do not survive and/or proliferate, thus making it difficult to retrieve them for morphologic evaluation. In order to be able to study sequential morphologic changes occurring *in vivo* in multiple aliquots derived from the same original cell population, we developed a technique which involves the "*in vivo* culture" of normal or preneoplastic cells on de-epithelialized tracheal stroma. The de-epithelialized stroma provides those factors required for the survival and differentiation of normal and preneoplastic epithelial

FIGURE 8. Generalized stratified metaplasia, after 2 weeks exposure to 200 μg DMBA. The surface cells contain Alcian Blue-positive material, indicative of the presence of acid mucosubstances. (PAS–Alcian Blue, pH 2.6, × 240.)

cells which are apparently lacking in the intramuscular or subcutaneous environment. The procedures have been published in detail (*36,48*). Briefly, fresh suspensions of normal respiratory tract epithelium or cultured epithelial cells originating from tracheas previously exposed to PAH *in vivo* are inoculated into the lumina of de-epithelialized tracheas. De-epithelialization is achieved by enzyme digestion (*36*) or by devitalization of

FIGURE 9. Focal atypical lesions observed after 24 weeks exposure to 200 μg DMBA. (Toluidine blue, Epon, × 300.)

the whole organ by repeated freeze-thawing (*48*). The tracheas are then transplanted into the dorsal subcutaneous tissue of host rats or nude mice. Immunocompetent hosts (rats) can be used for the growth of normal rat tracheal epithelial cells. Immunodeficient hosts (e.g., irradiated nude mice or thymectomized, irradiated rats) are generally required for long-term maintenance of preneoplastic and neoplastic cell lines. In the latter case, if immunocompetent hosts are used, the established tracheal lining rejected within 1 to 4 weeks of cell inoculation and implantation. Once inoculated tracheas are implanted into suitable hosts, approximately 2 to 3 weeks are required for the complete regeneration of a tracheal epithelium. When normal epithelial cells have been inoculated, mucociliary epithelium with occasional areas of simple or stratified squamous epithelium is seen. Inoculated preneoplastic cell lines give rise to a relatively homogeneous epithelia of either an atrophic, cuboidal, or stratified squamous nature, exhibiting various degrees of atypia (Fig. 10). After variable lengths of time during which the epithelium remains remarkably stable, some of the preneoplastic cell lines give rise *in vivo* to cell subpopulations which invade the tracheal wall. Tracheas inoculated with neoplastic cell lines are rapidly repopulated with what initially appears to be a stratified squamous epithelium exhibiting moderate to marked atypia. Within 1 to 4 weeks, one sees a rapid disorganization of this epithelium with marked cell proliferation and invasion of the transplant wall.

In general, the epithelial morphologies observed in these studies are highly reminiscent of various neoplastic and preneoplastic lesions observed following exposure of intact rat tracheas to PAH. This "*in vivo* culture system" appears to be well suited to the study of the growth and differentiation characteristics of carcinogen-altered or preneoplastic cell populations. This is otherwise not readily accomplished, since nonneoplastic cells when inoculated subcutaneously or intramuscularly either do not survive or do not proliferate and are thus often difficult to retrieve for morphologic evaluation.

This *in vivo* culture system has also been further developed for purposes of investigating differences in the stromal requirements, for growth and differentiation *in vivo*, or normal and PAH altered cell populations (*36*). For normal tracheal epithelial cell survival and maintenance of mucociliary differentiation *in vivo*, an intact but not necessarily viable stroma (tracheal, intestinal, esophageal, or bladder) is required. Preneoplastic and neoplastic cells will survive and differentiate *in vivo* on all substrates including, for example, dacron mesh which will not support normal cell growth and differentiation.

Repopulation of Tracheal Transplants with Human Epithelial Cells

Using the same repopulation techniques, it is possible to reconstitute a normal human respiratory epithelium in previously de-epithelialized rat tracheas transplanted sc in nude mice (*14*). Using cells derived directly from tissues obtained at autopsy, we have been able to establish up to 10 reconstituted tracheal transplants from each stillborn fetus. Within 2 to 4 weeks of cell inoculation and transplantation, these tracheas are fully repopulated with a normal appearing human respiratory epithelium. This epithelium is enzymatically harvested and inoculated into 30 to 50 new de-epithelialized rat tracheas and transplanted subcutaneously into a

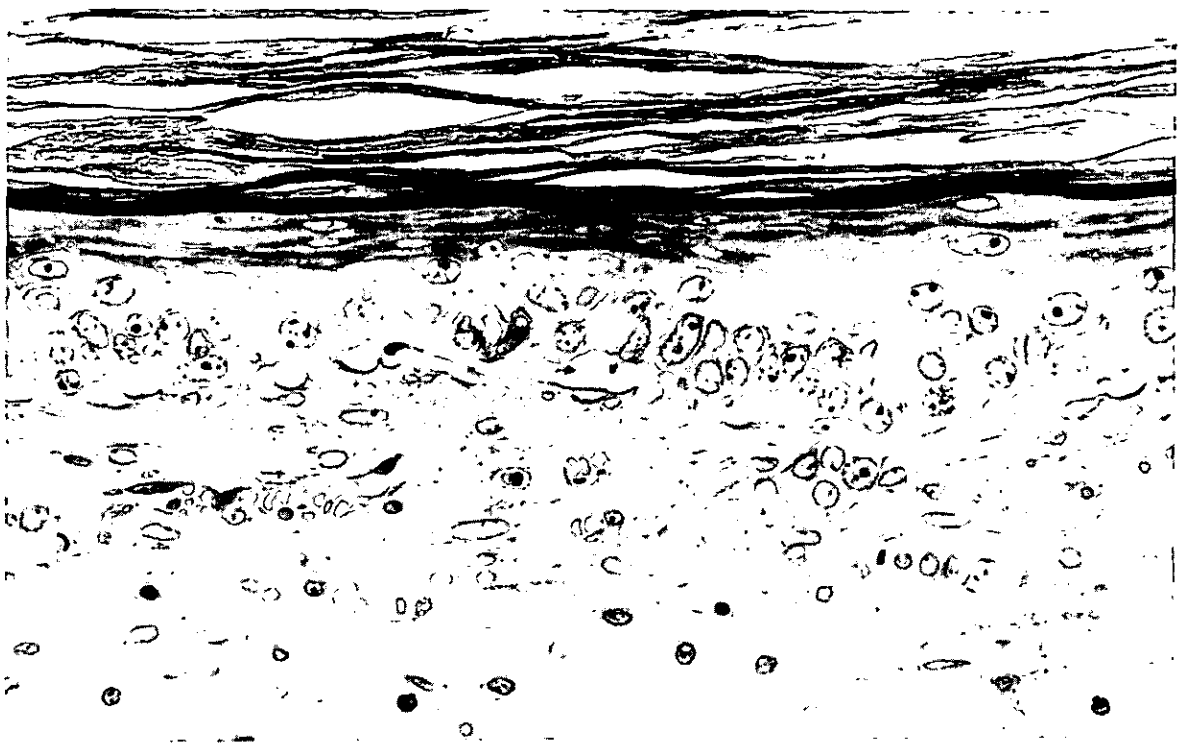

FIGURE 10. Reconstituted stratified squamous epithelium with mild atypia, as seen three weeks after inoculation of a "preneoplastic" cell line into denuded tracheas. (Toluidine blue, Epon, × 380.)

new series of host animals. In this way, with each sequential *in vivo* passage of human epithelial cells a population amplification factor of 3 to 5 is achieved. This procedure can be repeated at least four times (*49*). Preliminary experiments involving exposure of reconstituted human respiratory epithelium to DMBA suggest that studies similar to those carried out with exposed rat tracheal epithelium can also be carried out with human respiratory epithelium (*50*).

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